Protein degradation: **De-ubiquitinate to decide your fate**

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The ubiquitination/de-ubiquitination system that controls the degradation of many cellular proteins can be regulated at several of its distinct steps; one recently discovered control is important in *Drosophila* eye development.

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Current Biology 1996, Vol 6 No 6:662–665

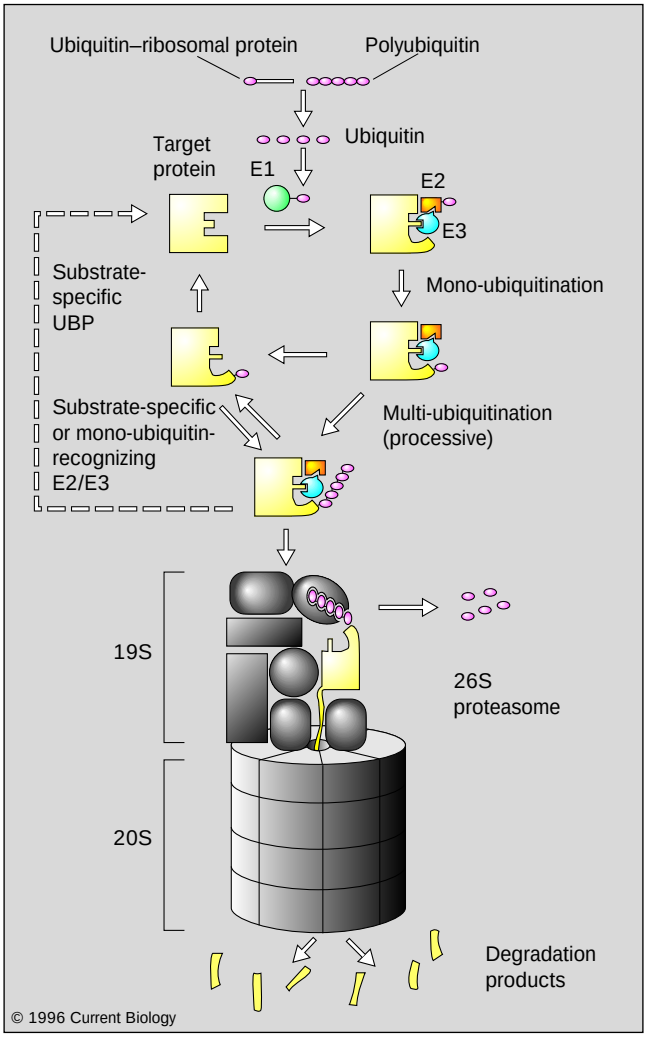
The covalent attachment of ubiquitin to proteins can act as a signal for their degradation by a multisubunit complex known as the 26S proteasome [1]. A combination of *in vitro* reconstitution studies and mutational analyses in yeast has identified many of the enzymes responsible for ubiquitination, and provided evidence for essential roles of this pathway in the proteolysis of damaged or mis-folded proteins, as well as in determining the high turn-over rate of naturally short-lived proteins. The large diversity of potential ubiquitination substrates presents a challenge to the recognition mechanism that is, in some cases, compounded by the need to regulate protein stability according to the cell cycle or environmental cues.

One possible solution to this problem would be for many substrates to share common degradation signals, but for their efficacy to be altered by substrate modifications such as phosphorylation or denaturation. Alternatively, the diversity of ubiquitinating enzymes may accommodate recognition of many different signals; in this case, the degradation of a single protein, or a small group of proteins, could be regulated according to the activity of a specific ubiquitinating enzyme. Whether de-ubiquitinating enzymes contribute to the regulation of protein degradation has remained speculative in the absence of demonstrated substrate specificity. However, recent studies by Fischer-Vize and colleagues [2] have now provided an example in which loss of function of a de-ubiquitinating enzyme is associated with a specific developmental phenotype, prompting a re-examination of how regulation and specificity are achieved in ubiquitin-mediated events.

Enzymology of ubiquitination

The ubiquitin pathway of protein degradation involves several steps catalysed by distinct enzymes (Fig. 1). Ubiquitin is a 76-amino-acid protein that is generated either by proteolysis of newly-synthesized polyproteins or by recycling ubiquitin molecules that have been linked to other proteins post-translationally. Free ubiquitin is activated by an 'E1' enzyme forming a thiol-ester between the carboxyl terminus of ubiquitin and a cysteine residue of E1, and is

Figure 1



The ubiquitin-dependent protein degradation pathway. Free ubiquitin molecules – generated from polyprotein precursors or protein conjugates degraded by the proteasome – are linked to target proteins by the action of E1, E2 and E3 enzymes. Multi-ubiquitination may be processive or interrupted and continued by the same or different E2/E3 enzymes. De-ubiquitinating enzymes (UBPs) can reverse the downward path toward degradation by the proteasome at any time, and mono-ubiquitinated proteins may be particularly accessible to recognition by substrate-specific de-ubiquitinating enzymes. Degradation of a protein may be modulated by ubiquitin concentration, changes in substrate conformation – induced, for example, by binding partners, phosphorylation or denaturation – that affect specific recognition signals, steric hindrance or flexibility, or by changes in activity of specific enzymes that catalyze ubiquitination or de-ubiquitination.

subsequently transferred to a cysteine residue of one of several 'E2' ubiquitin-conjugating enzymes. *In vitro*, some E2 enzymes directly catalyze the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the ϵ-NH2 group of an internal lysine residue of a protein substrate, whereas others require the participation of an 'E3' enzyme, often known as a ubiquitin ligase.

The best-characterized E3 enzyme, E6-AP, forms a ubiquitin–thiol-ester intermediate, but some other E3 enzymes may act simply by simultaneous binding of an E2 enzyme and a substrate molecule. Most commonly, only a single or small number of lysine residues of a substrate are ubiquitinated. However, multiple ubiquitin molecules are frequently conjugated to a single protein molecule by sequential attachment to an internal lysine (usually K48) of a ubiquitin molecule already linked to the substrate. Multi-ubiquitination increases affinity for the proteasome and is essential for protein degradation *in vivo*.

Recognition of ubiquitination signals

What are the primary recognition signals for protein ubiquitination and how are they recognized by E2/E3 enzymes? Experiments with model substrates defined the 'N-end rule' signal, where several destabilizing amino-terminal residues are recognized by a specific E3 enzyme (UBR1 in yeast). Few substrates appear to rely on this signal *in vivo*, however, as loss of UBR1 function in yeast eliminates degradation of model 'N-end rule' substrates without phenotypic consequence or a measurable effect on protein turnover.

A hypothetical degradation signal that is also potentially simple enough to be shared by many proteins is a 'PEST' sequence, a region rich in amino acids proline (P), glutamate (E), serine (S) and threonine (T) that is found at particularly high frequency in proteins with short half-lives. Transferrable ubiquitination/degradation signals have been identified within PEST regions of yeast proteins GCN4 and CLN3 [3] but, as yet, no specific consensus sequence has been found, nor has an E2 or E3 enzyme been identified that recognizes these sequences with high affinity. The paucity of obvious sequence similarity among the (few) degradation signals identified to date (in B-type cyclins, Jun, Mos, CLN3, GCN4 and MATα2) suggests the existence of a large number of structurally distinct recognition determinants, likely to exceed by far the number of E2 enzymes.

The current inventory of twelve E2 enzymes (UBC1-12) in yeast is likely to be fairly complete, because they are readily identified by sequence similarity over a 150-amino-acid domain and by their shared ability to be recipients of ubiquitin from E1 enzymes. The implication that a single E2 enzyme would have to recognize multiple substrates directly or by interaction with different E3 proteins is confirmed by studies with *ubc2* mutant yeast [4]. *UBC2* is required for normal sporulation, DNA repair (hence the gene's alternative name *RAD6*) and 'N-end rule' substrate degradation, but these activities are selectively dependent on UBR1 function ('N-end rule' degradation only) and the intact carboxyl terminus of UBC2 (both sporulation and 'N-end rule' degradation), suggesting that UBC2 functionally interacts with at least three different proteins.

The diversity of E3 proteins in a cell is hard to assess at present, as the two cloned prototypes, UBR1 and E6-AP, are not related in sequence, and the biochemical mechanism by which E3 promotes ubiquitination by E2 enzymes has not been well defined. The identification of at least ten human proteins that share sequences — 'hect' domains — around the catalytically active cysteine of E6-AP [5] suggests that the E3 family may be quite large, earmarking E3 enzymes as the principal key to substrate specificity. Combinatorial recognition of substrates by pairs of E2 and E3 enzymes has been suggested, but neither E6-AP nor UBR1 require E2 enzymes for target binding *in vitro*.

Multi-ubiquitination

The attachment of a single ubiquitin molecule is not necessarily a one-way ticket to Palookaville. *In vitro*, some E2/E3 enzymes catalyze processive multi-ubiquitination, whereas others (usually E2 alone) favor the attachment of one or a small number of ubiquitins, conceivably because multimeric E2/E3 complexes encourage processivity. If E2/E3 enzymes also dissociate from mono-ubiquitinated substrates *in vivo*, this would provide an ideal opportunity for a substrate-specific de-ubiquitinating enzyme to act in the absence of steric hindrance from bound E2/E3 enzymes or a large branched chain of ubiquitins.

Mono-ubiquitinated intermediates may be recognized by E2/E3 enzymes that are not specific to the particular substrate, as it is known that covalent linkage of ubiquitin to a stable model protein suffices as a signal for further ubiquitination and degradation *in vivo*, by a mechanism that requires yeast UBC4 activity [6]. If UBC4 (and the related UBC5 and UBC1) are commonly used for extending ubiquitin chains that were initiated by other enzymes, this could explain the widespread effects of mutations of *UBC4* family genes on the half-lives of most short-lived and abnormal proteins.

Protein degradation

The 26S proteasome includes a 20S catalytic core which forms a hollow cylinder lined with the active sites of proteases of various specificities, and a 19S particle which forms an ante-chamber to the narrow (1.3 nm) entrance of the 20S cylinder [7]. Binding of multi-ubiquitin to subunit 5 of the 19S particle targets proteins to the proteasome, where they are assumed to be unfolded by ATPases in

order to gain entrance into the 20S cylinder where they are cleaved into oligopeptides. Although entry into the proteasome normally signals the end of a protein's unique contribution to the cell, there are some important exceptions.

The cleavage of cellular antigens prior to presentation by class I major histocompatibility complex (MHC) molecules appears to involve the proteasome, and indeed the cleavage specificity can be modified by cytokine-induced changes in the representation of particular protease subunits. The proteasome can also catalyse partial proteolysis, for example degrading only the carboxyl terminus of the primary NF-κB translation product, p105, to leave an intact and functional p50 fragment. It is not known if this domain of the protein is spared because it cannot be unfolded, because it is firmly anchored in the 19S particle by ubiquitinated residues or for some other reason.

Regulation of ubiquitination

An emerging characteristic of ubiquitin-mediated degradation is that it is a regulatable process. For all proteins, misfolding and random or thermal denaturation are important autonomous signals for degradation. This may allow formation of a novel structure that acts as a specific degradation signal. Studies with model substrates, however, suggest that introduction of flexibility — for example, by addition of an unstructured 'linker' or reduction of disulphide bonds — can greatly enhance the recognition of a pre-existing degradation signal. E2/E3 enzymes may be designed to demand substrate flexibility for optimal binding, or flexibility may enhance access of bound E2/E3 enzymes to internal lysine residues of the substrate. In similar fashion, proteins that cannot bind their usual cellular partners, because of denaturation, mutation or regulation, may be particularly prone to E2/E3 recognition simply from the absence of steric hindrance. Such substrate-mediated cues can be supplemented in response to general signals such as heat-shock by the induction of ubiquitination components that also act generally, such as the multi-ubiquitin protein and UBC4/UBC5 enzymes.

But what of more specific signals that are designed to affect the ubiquitination of only single or a small number of proteins? These signals must impinge either on the substrate directly or on components of the ubiquitin pathway that are selective in their action. Such enzymes need not be rigorously substrate-specific — they may be like the several protein kinases that are known to transduce a signal through a specific substrate, while also enhancing the phosphorylation of other proteins without detrimental effects.

For ubiquitination, there is evidence for both types of regulation in response to cell-cycle cues. An E3-containing complex, known as the cyclosome, promotes ubiquitination of cyclin B *in vitro* only if isolated from late M-phase extracts of *Spisula* (clam) embryos or *Xenopus* eggs. The complex can be inactivated by an okadaic-acid-sensitive phosphatase and re-activated by cyclin-dependent kinase phosphorylation [8]. By contrast, the effects of cyclin-dependent kinase activity on promoting the ubiquitin-mediated degradation of a G1 cyclin, CLN3, in yeast appear to be mediated, at least in part, by phosphorylation of a specific residue of CLN3 embedded in a PEST sequence that can act as a transferrable degradation signal [3].

The regulation of ubiquitination by signal-induced substrate phosphorylation may be quite widespread. Autophosphorylation of Mos at serine 3 occurs during the latter stages of *Xenopus* oocyte maturation, and appears to be essential for stabilization against ubiquitin-mediated degradation. Conversely, Iκ-Bα — a negative regulator of NFκB that sequesters the transcription factor in the cytoplasm — undergoes signal-dependent phosphorylation at serines 32 and 36 as an essential pre-requisite for subsequent ubiquitination and proteasome-mediated degradation.

Activation of Iκ-Bα phosphorylation by a partially purified 700 kD protein kinase complex *in vitro* requires the addition of ubiquitin, E1 and a catalytically active E2 enzyme of the UBC4 class, the same E2 enzyme that subsequently participates in the ubiquitination of phosphorylated Iκ-Bα *in vitro* [9]. The ubiquitination target is not known, but the proteasome does not participate in the activation event. Thus, an apparently straightforward instance of regulation of ubiquitination by substrate modification may yet be overturned in favor of a ubiquitination enzyme as the primary focus of regulation. This example, and the ligand-induced ubiquitination and subsequent endocytosis of STE2, the yeast α-factor receptor, which again appears to depend on phosphorylation [10], also emphasize that ubiquitination need not serve only as a tag for binding to the proteasome.

De-ubiquitination

So where do de-ubiquitinating enzymes come in? Although a large number of these enzymes (at least 15 in yeast) have been identified biochemically or by sequence motifs, mutational phenotypes in yeast and direct biochemical characterization have so far provided little evidence of substrate specificity. Some of these enzymes can only cleave ubiquitin from precursor-like fusion proteins, but several also have isopeptidase activity that in one case (isopeptidase T) recognizes only free multi-ubiquitin chains, but which more often can act on either multi-ubiquitinated or mono-ubiquitinated proteins without strict specificity toward the non-ubiquitin moiety.

Four of the first five yeast de-ubiquitinating enzymes to be identified can be mutated simultaneously without

obvious effects on ubiquitin-mediated protein degradation or other phenotypes [11]. Mutations in the fifth protease, DOA4, do impair growth but, perhaps counter-intuitively, they reduce degradation of model substrates [12]. The DOA4 protease appears to be required for cleavage of ubiquitin from short peptides during the final steps of protein degradation and it was postulated that accumulation of unprocessed peptide conjugates impairs access of protein–ubiquitin conjugates to the proteasome. This argument can be extended to the generalization that proteasome-bound de-ubiquitinating enzymes are likely to have unique functions by virtue of their localization, whereas soluble cytosolic enzymes may have redundant functions unless they have distinct substrate specificities. Enter *fat facets*.

The *Drosophila* Fat facets protein shows similarity to other de-ubiquitinating enzymes in two small regions surrounding critical cysteine and histidine residues, and can cleave a model ubiquitin fusion protein in *Escherichia coli* [2]. Site-directed mutation of the critical cysteine and histidine residues showed that this activity was essential to its normal function in *Drosophila*. In the absence of *fat facets* (*faf*) function, flies survive to adulthood but their eyes develop abnormally, incorporating supernumerary cells that undergo neuronal differentiation into each ommatidial cluster. Also, adult females are infertile.

In contrast to the loss of DOA4 in yeast, loss of Fat facets in *Drosophila* is suggested to promote protein degradation, as the rough-eye phenotype is partially suppressed by reducing the dosage of a proteasome component. The simplest interpretation of these results is that Fat facets normally prevents a specific protein from entering the degradation pathway. If so, one might wonder if Fat facets simply returns the putative substrate to its original state, by removing its ubiquitin tag, or whether the substrate is modified by its experience, perhaps retaining some particular configuration of ubiquitin residues that alter its activity without demanding its degradation.

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